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13. ABSTRACT *(Maximum 200 words)*

184A1 cells do not form polarized, glandular epithelial structures when plated onto Matrigel, nor do they polarize if plated onto Transwell tissue culture inserts; therefore, 184A1s could not be used to address the primary aim of my proposal: to determine if the loss of receptor system polarization provides a growth advantage, enhances motility, or changes the differentiated state of normal cells. However, because the growth and motility of 184A1 cells require EGFR stimulation and because EGFR mis-regulation is implicated in the progression of human breast cancer, the 184A1 cell system still represents a excellent cell system in which to investigate EGFR regulation. My work has revealed that the constitutive endocytosis of the empty EGFR can be an active and specific form of EGFR spatial regulation in 184A1 cells. The process requires formation of coated pits, is specific and saturable for the EGFR and is upregulated at high cell densities. As a result of the rapid constitutive endocytosis, there is a significant increase in the proportion of receptors that can be found within the cell. Mounting evidence suggests that activated receptors initiate different signalling pathways if they are localized to vesicles as opposed to plasma membrane associated. Rapid constitutive endocytosis may provide a mechanism by which a large pool of receptors is made available to initate, preferentailly, signals from internal vesicular membranes. Endocytosed ligands, or internalized, pre-activated EGFR or receptor family members could activate the internal pool of receptors either directly or in trans, respectively.

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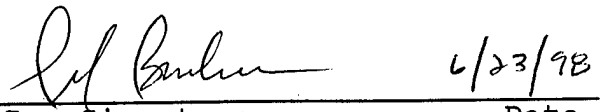

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INTRODUCTION:

Special Distribution of the EGF Receptor System in the Regulation of Breast Epithelial Cell Growth and Organization

The epidermal growth factor receptor (EGF-R) system is necessary for the motility, proliferation and differentiation of human mammary epithelial cells (HMECs) *in vitro*. Additionally, the EGF-R system displays a highly organized spatial distribution *in vivo*. Because the EGF-R system plays a central role in HMEC proliferation and cell motility, it is reasonable to suspect that any defects in its regulation could lead to the clonal expansion of 'premalignant' cell populations. Such expanding clonal populations could give rise to cancerous clones and ultimately metastatic disease. In general, the receptor is subject to two forms of negative regulation, covalent and spatial. Here, I investigate how spatial regulation of the EGF-R influences the physiology of human mammary epithelial cells (HMECs). Epithelial polarization is a form of spatial regulation that effects the function of many molecules including the EGF-R. The spatial distribution of the EGF-R system is highly organized in breast epithelium *in vivo*. *In vivo*, the receptor and one of its ligands, transforming growth factor alpha (TGF–alpha), are localized to the basolateral surface of mammary alveolar structures; on the other hand, epidermal growth factor (EGF) is synthesized and secreted from the apical side into the lumen of the alveoli. Negative regulation of the EGF-R system could be disrupted by removing the spatial restrictions which segregate one of the ligands from the receptor and/or the receptor from second messenger systems. Down-regulation is another form a spatial regulation where by both surface receptor numbers and total receptor mass are proportionately reduced in response to ligand availability, presumably attenuating signaling by reducing the number of receptors available at the surface. Here I investigate the spatial regulation of both the EGF-R and its ligands during HMECs growth and differentiation and determine the consequences of disrupting these regulations.

SPECIFIC AIMS:

1. Define the spatial distribution and expression levels of the EGF-R system in proliferating and spatially organized normal HMECs.

2. Determine whether a loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage, enhances cell motility, or changes the differentiated state of normally organized epithelial cells.

STATEMENT OF WORK:

PART I

Define the spatial distribution and expression levels of the EGF-R system in proliferating and spatially organized normal HMECs. (Months 1-18).

a. Determine the expression levels of the EGF-R and it ligands in normal HMECs. (Months 1-6).
b. Determine the spatial distribution of the EGF-R and it ligands in the organotypic structures of normal HMECs. (Months 7-18).

PART II

Characterize the molecular trafficking mechanism which redistributes the unoccupied EGF-R into intracellular pools. (Months 19-36).

a. Are internal EGF-R rapid endocytosed and recycled?

b. Are the receptor pools stationary and segregated from the surface population of EGF-R?

c. Define the general endocytic properties of 184A1 cells.

d. Define the biochemical requirements for the establishment of the intracellular EGF-R pools.

BODY OF REPORT:

The goal of my proposal was to investigate how disrupting the polarization of the EGF receptor system (the receptor plus ligands) would effect human mammary epithelial cell physiology. The EGF-R system displays a highly organized spatial distribution *in vivo*. The receptor and one of its ligands, transforming growth factor alpha (TGF–alpha), are localized to the basolateral surface of mammary alveolar structures, and, epidermal growth factor (EGF), an additional ligand, is synthesized and secreted from the apical side into the lumen

of the alveoli (11). When I began my work, I wanted to know if the mislocalization of either the receptor or any of its ligands to the inappropriate membrane domain would impart to the cell disregulated growth, enhanced motility or other cell-physiological changes. I was unable to identify a cell system that would allow me to address the central goal of the proposal, however, the cell line with which I began my work, 184A1, unexpectedly revealed that the constitutive endocytosis of the empty EGF-R can be a very active and specific form of spatial regulation. 184A1 cells were isolated by Martha Stampfer as an immortalized derivative from the parental 184 cell line (15). Importantly, the proliferation of 184A1 is absolutely dependent upon EGF-R stimulation (annual report 1997, fig. 2)(14); also, 184A1 cells respond motogenicly to EGF-R stimulation (8) and organize into organotypic structures in the presence of EGF when plated onto the extracellular matrix material Matrigel (annual report 1997, fig 3). The responsiveness of 184A1 cells to EGF-R stimulation makes it a very attractive model in which to investigate EGF-R regulation. For these reasons, and because the endocytosis of the empty EGF-R has largely been ignored, I focused my efforts toward characterizing this form of spatial regulation.

Although there are suggestions in the literature that primary hepatocytes (5, 6) rapidly endocytose empty EGF-R, most other cells types, including another human mammary epithelial cell line, HB2, maintain a majority of the receptor population at the cell surface. HB2 cells were isolated by Joyce Taylor-Papadimitriou (2). I have included HB2 in many of my investigations. Like 184A1, HB2 express large numbers of EGF-R, but unlike 184A1, HB2 do not respond in any obvious way to EGF-R stimulation (annual report 1997, fig. 2). As shown in previous annual reports, 184A1 can internalize the unoccupied EGF-R almost as efficiently if not as efficiently as the ligand occupied EGF-R (annual report 1997, fig. 6; shown again here in fig. 1). I have also shown that unoccupied EGF-R is rapidly recycled (annual report 1997, fig. 11), and that the endocytosis of the empty receptor produces a steady state situation in which a large fraction (30-40%) of the total

receptor pool can be found within internal vesicles (annual report 1997, fig. 7 & 9). 184A1 do not endocytose the transferrin (Tf-R) or fluid phase markers any more rapidly than HB2 cells, therefore, at least at one level the rapid endocytosis of the empty EGF-R in 184A1 is specific (annual report 1997, fig 10). I have reported all these findings previously and they are currently being submitted for publication.

During this last year, I have attempted to better characterize the mechanisms that regulate the endocytosis of unoccupied EGF-R in 184A1. I have found that the constitutive endocytosis EGF-R does not depend upon tyrosine phosphorylation, is saturable, and is specifically upregulated as a function of cell density. Also, for reason discussed below, I had hypothesized that rapid recycling of the unoccupied receptor would be interrupted by the activation of surface receptors. However, my preliminary results, reported below, do not suggest this.

In the usual paradigm, EGF-R trafficking is initiated by ligand binding, activation of receptor's intrinsic tyrosine kinase activity, and ultimately phosphorylation of internal tyrosines which induce conformational changes in the receptor transforming it into a substrate for endocytosis (18, 19). Tyrosine phosphorylation of the receptor is central to the model. Tyrosine phosphorylation of the receptor is easily measured and indicates if receptor has been activated. In order to be sure that we were not somehow specifically inducing activation of the receptor in 184A1 cells with our antagonistic antibody (mAb 225) or that somehow EGF-R were being maintained in an endocyticly activated form in 184A1, we performed two experiments. First, I measured the steady state level of phosphotyrosine associated with EGF-R in 184A1 and HB2 in the presence and the absence of exogenous EGF (fig 2A). In the absence of EGF, both 184A1 and HB2 had low levels of phosphotyrosine associated with the EGF-R, but both levels were quite low, and the levels were similar between the two cell types. Next I measured the induction of tyrosine phosphorylation in 184A1 after the addition of EGF or mAb 225 (fig. 2B). In order to be thorough, a baseline measurement was once again made in the absence of any exogenous ligand, and, for the purpose of comparison,

EGF was added at two different concentrations (1ng/ml and 100 ng/ml). The mAb 225 was added at 1μg/ml. Again low constitutive levels of phosphotyrosine can be seen associated with the receptor and the addition of EGF increases that level significantly in a does dependent way. Importantly, mAb 225 did not, even to the slightest degree, induce the tyrosine phosphorylation of the EGF-R.

It has been reported that ligand induced endocytosis of the EGF-R is a saturable process (17). In other words, as more and more ligand is added to cells the efficiency of endocytosis decreases. I wanted to know if the same property could be true of the constitutive endocytic process. In order to measure ligand induced saturation of receptor endocytosis one measures the efficiency of endocytosis over a range of ligand concentrations. However, in order to measure the saturation of the constitutive process, it is necessary to have increasing numbers of unoccupied receptors. To this end, I set out to overexpress the EGF-R in 184A1. I transduced 184A1 cells with a selectable retrovirus that is engineered to express the human EGF-R. Transduced clones were selected in the presence of 300 mg/ml G418, and individual clones were isolated and expanded. Overexpression of the receptor was measured by western blotting (fig. 3) and radioactive ligand binding assays (data not shown). It was clear from the results that all six clones that were tested overexpress EGF-R. I then measured the efficiency of endocytosis of the unoccupied receptor as a function of receptor levels (fig. 4). As predicted, if the process was saturable, the efficiency of empty receptor endocytosis decreased as receptor numbers increased. Thus, the process of constitutive endocytosis of EGF-R in 184A1 is saturable.

Throughout the course of my work with 184A1 cells, I have observed that the absolute rate of endocytosis of the empty EGF-R can vary (fig.1 or annual report 1997 fig. 6). Despite the variations, the constitutive endocytosis of the EGF-R in 184A1 was always greater than that measured in HB2. Similarly, variations have been reported for the efficiency ligand induced endocytosis, yet there are no published explanation for the variation (18, 19). Several experiments I performed

suggested to me that cell density was influencing the rate of constitutive endocytosis. I tested this directly by measuring the efficiency of ligand induced and ligand independent endocytosis in 184A1 and HB2 as a function of cell density (fig. 5). As shown in figure 5 the only endocytic process that was influenced by cell density was that of constitutive endocytosis in 184A1 cells. Constitutive endocytosis of the EGF-R was unaffected in HB2 and in neither cell type was EGF endocytosis influenced by cell density. Despite the obvious effect of cell density upon constitutive endocytosis, there was still variation within this regulatory context. On different days, over different ranges of cell density, I see varying absolute degrees of variation across the range of cell densities tested. Thus, I have not identified all of sources of variation and regulation influencing the absolute rates of constitutive endocytosis, but I have identified one.

During the last year, I have also exerted a significant effort toward understanding how recycling of the unoccupied EGF-R might be regulated. I previously observed that 184A1 cells lose surface expression more quickly than HB2 cells (fig. 6). This finding was contrary to my expectations. I expected surface expression to be prolonged in the presence of ligand because I expected the large pool of recycling EGF-R to replace those that were being removed by the presence of ligand. Instead, I found that surface receptors were removed more rapidly in 184A1 than HB2. It should again be noted that HB2 and 184A1 express similar numbers of receptors and that the efficiency of ligand induced endocytosis is similar in the two cell types, therefore, I could not account for the difference based on those parameters. Based on these findings, I reasoned that receptor activation at the cell surface would interrupt recycling of the internally localized, empty receptors. Measurement of such regulation is not trivial. One must specifically label endocyticly localized receptors within the cell and follow their return to the cell surface. To accomplish this, I developed a new technique. I biotinylated mAb 225 via a linker that contained a disulfide bridge (Pierce cat no. 21331) . I allowed 184A1 cells to internalize the biotinylated mAb 225 and come to steady state where

a significant proportion of the mAb 225 is now within the cells (annual report 1997, fig. 7). I removed the biotin from the cell surface associated mAb 225 with a buffer that contained 50 mM glutathione, and then I followed the reappearance of biotin to the cell surface with ^{125}I-streptavidin in the presence or absence of EGF. Unexpectedly, I found that addition of EGF did not interrupt recycling of empty receptors (fig. 7). The experiment has only been done once, so I consider the result very preliminary. One concern is that I am not effectively activating surface receptors. The protocol involves incubating cells in the presence of an antagonistic antibody, mAb 225. I strip only the biotin from the surface of the cells, not the antibody (stripping of the antibody is very problematic and has been tried under various conditions); thus when EGF is added during the experiment the normal compliment of receptors is not available for ligand binding and activation. To address this possibility, I did performed one experiment that directly measured the activation of EGF-R during the course of such a protocol (fig. 8). Following the treatment of mAb 255, I detected some activation of EGF-R, as determined by tyrosine phosphorylation, but it was much less than the level of activation in the absence of any mAb 255. Consequently, I am hesitant to conclude that activation of surface receptors does not regulate recycling, however, that would be the best supported conclusion at this time. There must be another explanation for the apparent rapid disappearance of surface receptors in 184A1.

I am continuing to determine both the specificity and the regulation of constitutive EGF-R endocytosis in 184A1 cells. I expect at least two publications from this work. I will probably not be able to experimental address the functional consequences of such a form of spatial regulation in my thesis work, however, one can imagine how having a large pool vesicularly localized receptor might alter signaling from the EGF-R. It has been suggested that vesicularly localized EGF-R initiate different signalling pathways than receptors associated with the plasma membrane (1, 16). Rapid constitutive endocytosis may provide a mechanism by which a large pool of receptors is made available to initiate, preferentially, signals

from internal vesicular membranes. We know that endocytosed ligands, particularly TGF-alpha, a ligand made by 184A1 cells (annual report 1996, fig. 13), readily disassociates from EGF-R in the endosome (4). If additional receptors are present within an endosome, they then might be activated by the free ligand, thus, in such a scheme, there would be more activated EGF-R per ligand molecule within the cell than on the surface. Interrupting the recycling of unoccupied EGF-R by surface activation would further assure that more internal EGF-R were activated per ligand molecule. Ligand disassociation is not necessary consideration for such a model. EGF-R readily forms homo and heterodimers with other receptor family members (3, 7). Trafficking, activated family members may be sufficient to activate the internal pool of EGF-R in trans and thus changing the signaling balance. My suggestions are only speculative and other functions for internal pools of receptors or rapid constitutive endocytosis are possible. My findings may be an important consideration when thinking about both about EGF-R regulation and human mammary epithelial cell physiology.

CONCLUSIONS

1) The unoccupied EGF-R is rapid endocytosis by responsive human mammary epithelial cells.

2) Induction of tyrosine phosphorylation of the EGF-R is not necessary for rapid constitutive endocytosis.

3) Constitutive endocytosis of the EGF-R is upregulated in responsive HMECs as a function of cell density.

4) Constitutive endocytosis of EGF-R is a saturable process.

5) Recycling of the unoccupied EGF-R is not regulated by activation of surface EGF-R.

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Figure 1

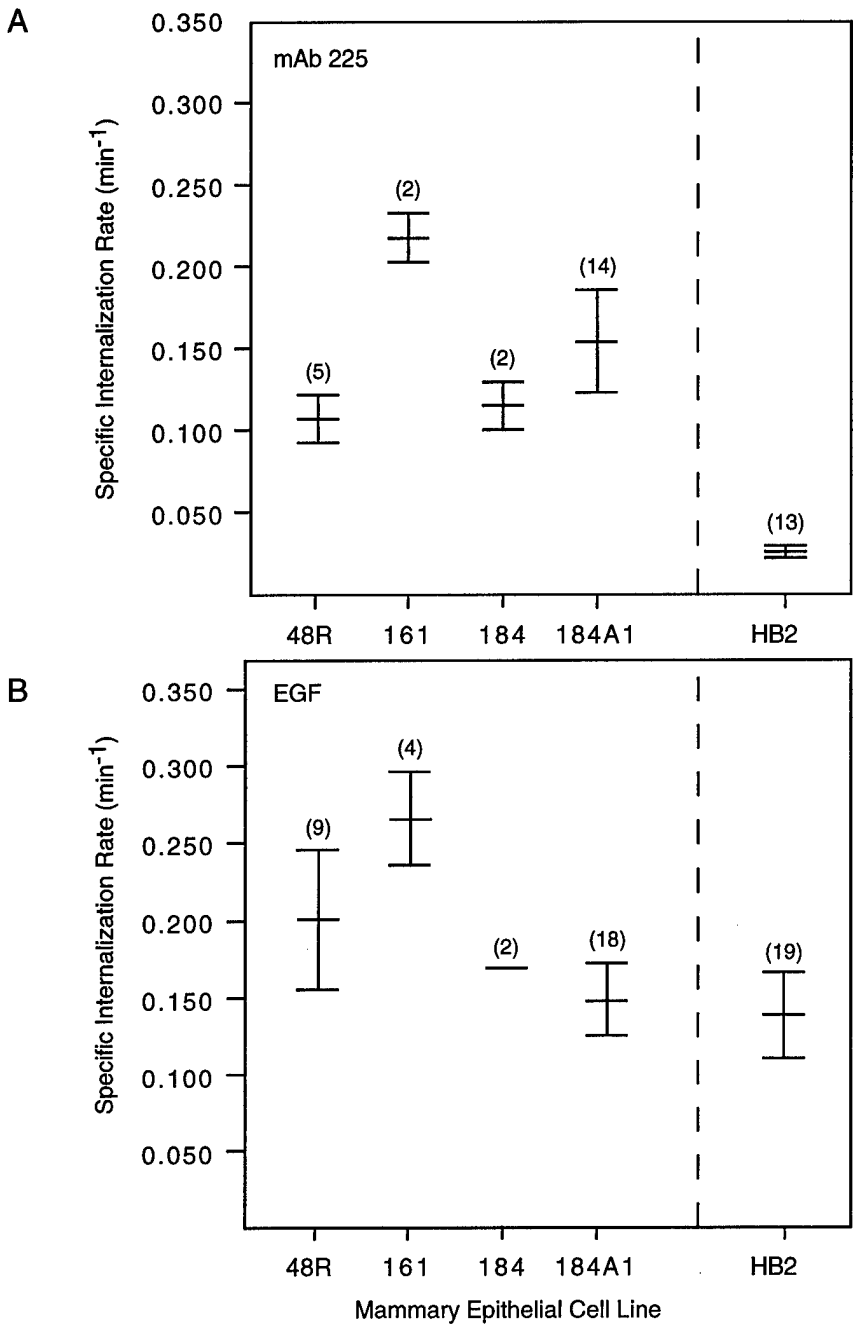

Figure 1. **Ligand induced endocytosis and constitutive endocytosis in HMEC.** Statistical summary of the internalization rates of both occupied and empty EGF receptors in different HMEC cell lines. The 184, 161, and 48R cells lines are primary cell lines derivered from reduction mamoplatsy (12). The 184A1 cell line is an immortal derivative of the 184 line(13). The kinetics of internalization were measured for 5 minutes using either (A) ^{125}I-mAb 225 or (B)^{125}I-EGF at concentrations from 0.20 nM to 0.50 nM to follow occupied EGFR. The relative distribution of label bewtween the surface and the inside of the cells was determined and converted to internalization plots as described (9, 10). The *wiskers* enclose all the data values within one standard deviations. Numbers in parentheses indicate the total number of independent experiments.

Figure 2

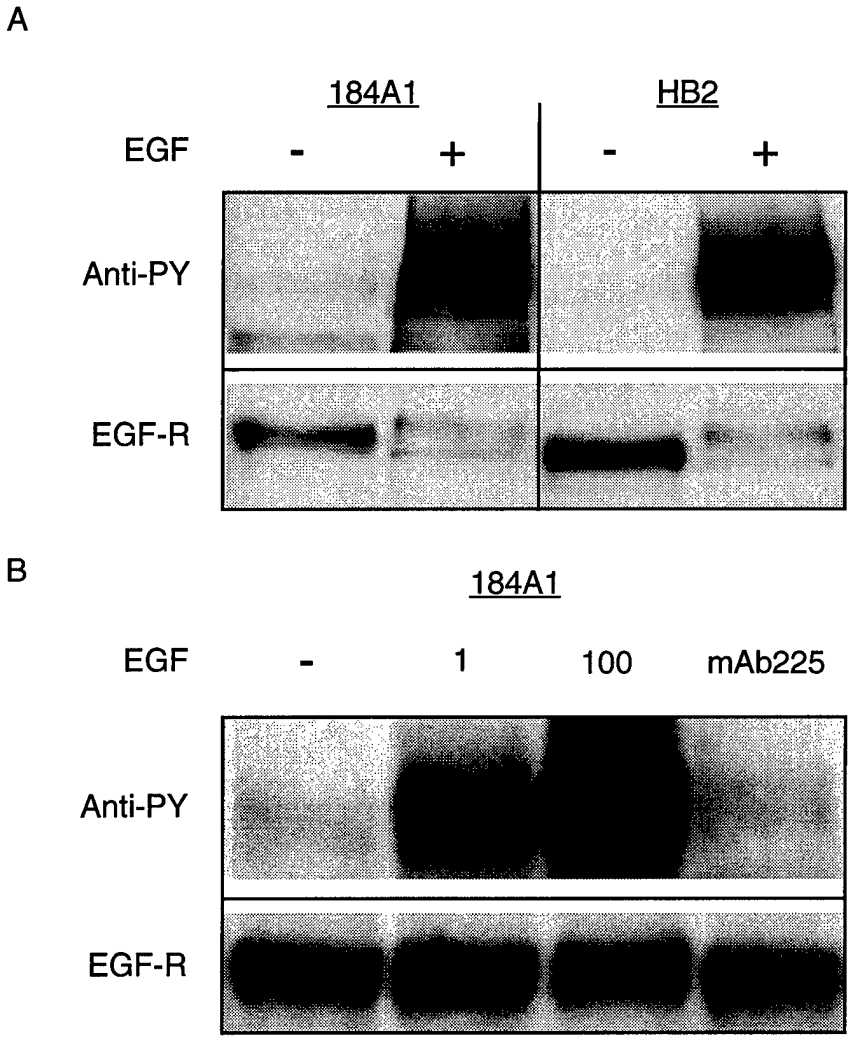

Figure 2. **Antagonistic mAb 225 does not activate the EGFR**. Immunoblot of EGFR and EGFR phosphotyrosine in 184Al and HB2 cells in the absence and presence of EGF. (*A*) Cells were grown to near confluency, and then either left untreated or were treated with 17 nM EGF for 10 min at 37°C. (*B*) Cells were grown to near confluency, and then either left untreated or were treated with either 0.17 nM or 17 nM EGF or 6.5 nM mAb 225 for 10 min at 37°C. Cells in (A) were extracted in a TritonX-100 (containing 1 µg/ml pepstatin, chymostatin, leupeptin, aprotinin, and 0.1 mM orthovanadate) those in (B) were extracted in RIPA (150 mM NaCl, 1% NP-40, 0.5% SDS, 0.5% DOC, 50 mM Tris pH 7.2, containing 1 µg/ml pepstatin, chymostatin, leupeptin, aprotinin, and 0.1 mM orthovanadate).

Figure 3

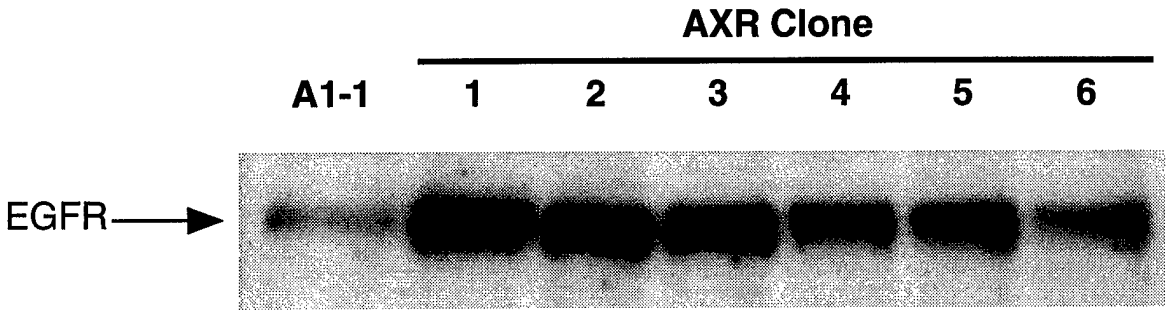

Figure 3. **AXR Clones #1-6 over-express the EGFR.** 184A1 were transduced with a retrovirus that was engineered to express human EGFR (AXR clones). The retrovirus also carries the selectable marker for G418 resistance. Transduced clones were selected in the presence of 300 mg/ml G418; individual clones were isolated and expanded. Over-expression of the receptor was measured by western blotting and radioactive ligand binding assays (data not shown). For western blotting, cells were grown to near confluence, samples were extracted in RIPA, normalized for cell number by counting cells on duplicate plates, separated by standard SDS-PAGE techniques, blotted and ultimately detected with polyclonal ant-EGFR antibody, N13, and radioactive protein-A.

Figure 4

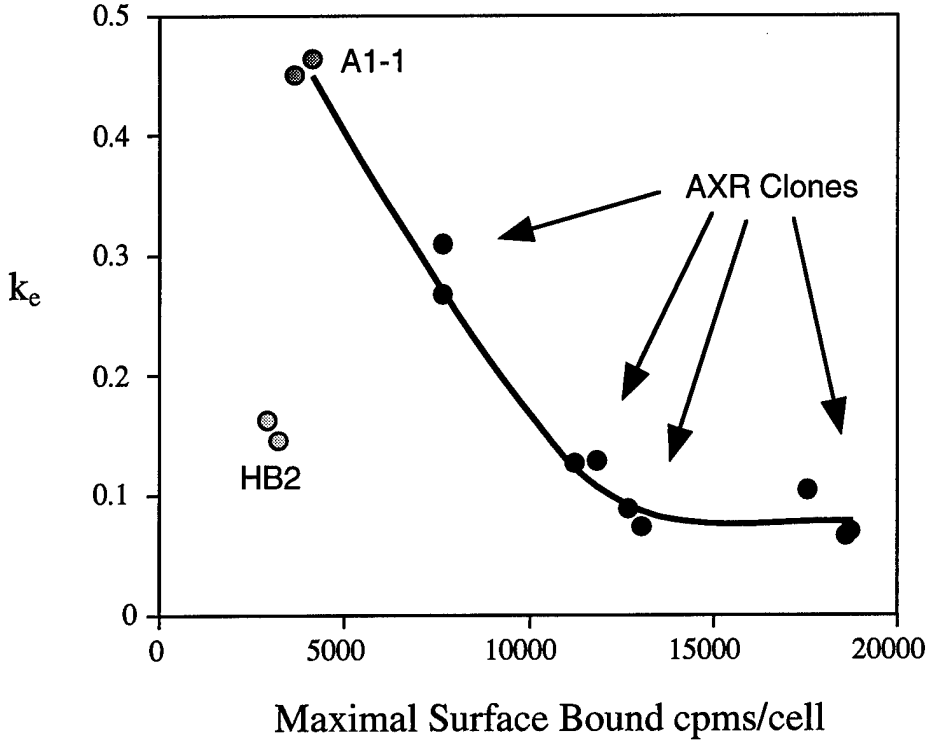

Figure 4. **The efficiency of constitutive endocytosis decreases in 184A1 as a function of receptor expression.** The kinetics of internalization of the unoccupied EGFR were measured for 5 minutes using ^{125}I-mAb 579 (like 225, another antagonistic EGFR antibody). The relative distribution of label bewtween the surface and the inside of the cells was determined and converted to internalization plots as described (9, 10). The protocol was such that mAb 579 was in excess, as a result, the amount of radiolabled ligand bound to the plate at any time point was directly propotional to the number of receptors per plate. After dividing the number of cpms by the number of cells per plate -- by counting the cells on a duplicate plate -- a direct, relative, determination of receptor number could be made and the relationship between receptor number and endocytic efficiency could be plotted (above).

Figure 5

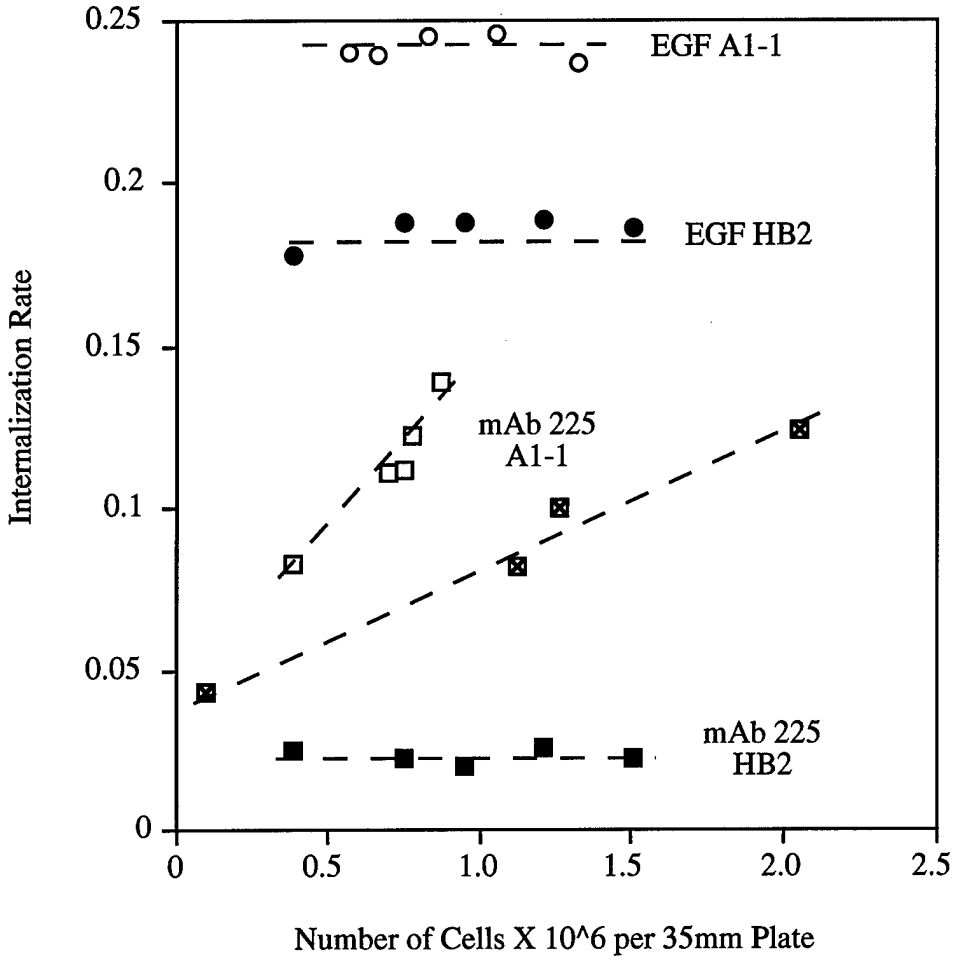

Figure 5. **The internalization of the empty EGFR in 184A1 is the only activity that is sensitive to cell density.** 184A1 and HB2 were plated at different densities and the efficiency of endocytosis of the empty and occupied EGFR was measured with ^{125}I-mAb 225 and ^{125}I-EGF respectively. The relative distribution of label bewtween the surface and the inside of the cells was determined and converted to internalization plots as described (9, 10). Two plots from two different experiments are shown for 184A1 constitutive endocytosis to show that even though there is a strong cell density effect that it can be variable.

Figure 6

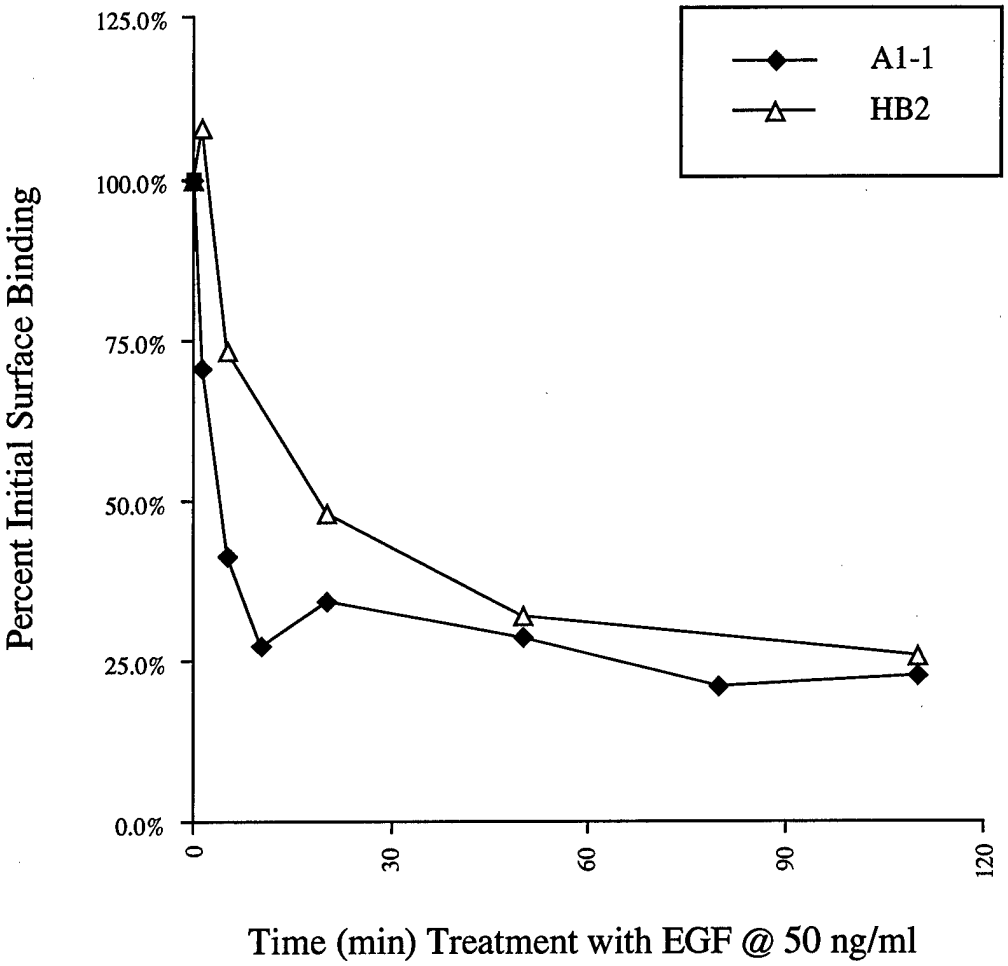

Figure 6. **Surface receptors are lost more rapidly from 184A1 than HB2 after expose to 50 ng/ml EGF.** 184A1 and HB2 cells were treated with 50 ng/ml EGF over a time course of 90 min. At specific time points cells were cooled to 4 °C to stop endocytosis, rinsed with cold PBS, and incubated 3 hour at 4°C in the presence of ^{125}I mAb 13A9 with binds EGFR in the presence or absence of bound ligand. Surface bound 13A9 collected and quantitated by gamma counter. The percentage of inital bound 13A9 was plotted over the time course and the results are shown above.

Figure 7

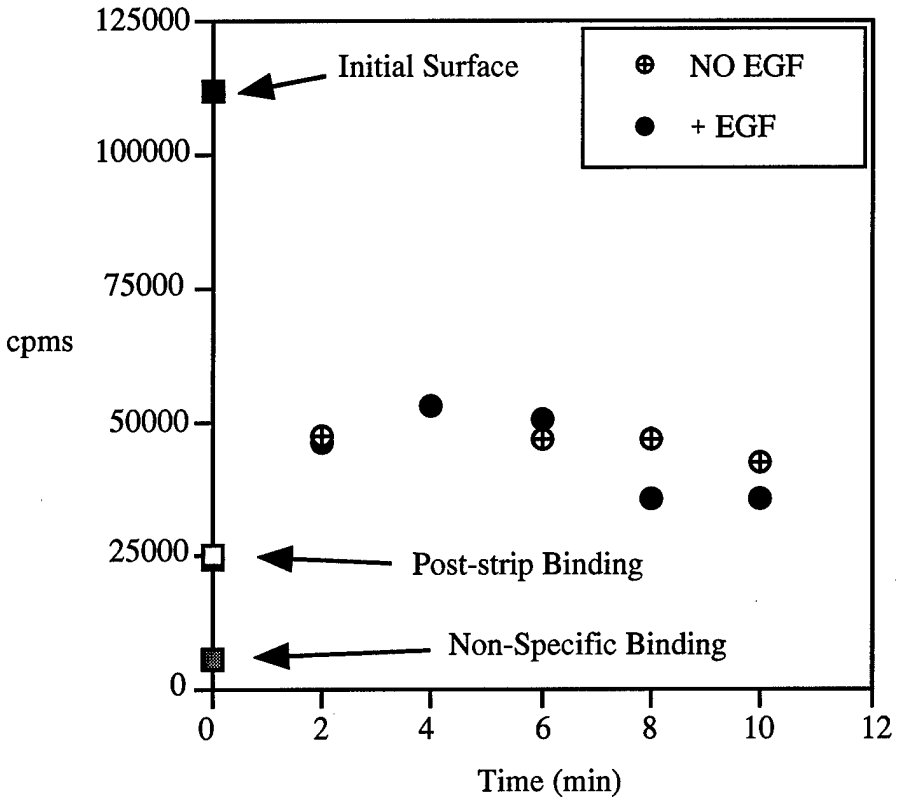

Figure 7. **Addition of EGF does not interupt recycling of empty receptors in 184A1.** 184A1 were allowed to internalize biotinylated mAb 225 for 2 hours at 37°C, thus allowing then to reach a steady state inside surface ratio (annual report 1997, fig 7). Cells were cooled to 4°C and biotin was stripped from cell surface assoicated 225 with 50 mM glutathione. Cells were then rapidly warmed to 37°C with media either in the presence or absence of 300 ng/ml EGF. At specific time points cells were cooled to 4°C, washed with PBS, and the amount of cell surface associated biotinylated mAb 225 was detected and measured with ^{125}I Strepavidin.

Figure 8

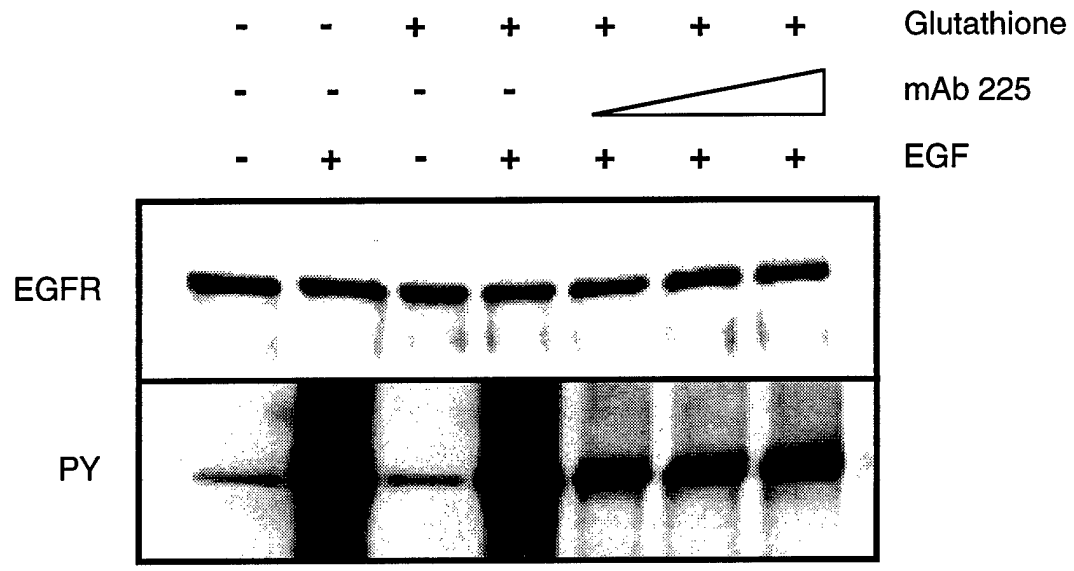

Figure 8. **Activation of EGFR is partially inhibited by the experimental protocol used in the recylcing experiments**. Cells were treated with or with 300 ng/ml EGF for 15 min either before or after 30 min at 4 °C in the 50 mM glutathione stripping solution. Cells treated with mAb 225 were grown in the presence of the antibody for 2 hours prior to treatment with glutathione -- as they would be in the experiments investigating recycling. All cells were extracted in RIPA, immunoprecipitated with anti-EGFR antibody 13A9, separated by SDS-PAGE, transblotted to nitrocellulose, and detected with either anti-EGFR polyclonal antibody N13 or RC-20 antiphosphotyrosine antibody (Signal Transduction Labs), followed by visulaization with enhanced chemiluminesence (NEN).

List of Personnel Supported by Grant

Patrick M. Burke	Graduate Student (PhD Candidate)	1994-1997

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